Adaptation of Influenza B Virus to Mice by Use of Certain Adjuvants.* (18181)

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Variations in influenza viruses have been studied by a number of investigators(1). Hirst(2) found that strains of influenza A virus isolated in the embryonated egg multiplied readily in mouse lung, and reached a high titer. Early passages were unaccompanied by any visible lethal or pathogenic effects, although after 4 or 5 passages visible reactions occurred. He could not show such adaptation to the mouse with influenza B virus. Likewise, comparing influenza A and B strains from 3 epidemics, he found(3) B strains unstable on freezing and storing at low temperatures. Irregular and spotty virus titers, rapid adaptation to the amniotic sac, and failure to adapt to mice without prior ferret passage were additional characteristics. Sugg (4) studied an egg-adapted strain of influenza A prime virus from which mice died rapidly with complete pulmonary consolidation after inoculation of allantoic fluid. However, lung suspensions did not produce pulmonary lesions on passage. This virus was readily propagated in mice, developing a high degree of virulence on serial passage. He suggested that virus multiplication in the mouse lung was due to a variant of the egg strain.

In view of the fact that influenza is often accompanied by secondary bacterial infections, it was thought that the addition of bacterial cells or cell extracts to virus preparations of low virulence might enable the virus to increase in virulence and establish itself in the experimental animal. Results of such an adaptation procedure are reported here.

Materials and methods. The influenza virus used in this study was a B strain (No. IB45) which had been isolated in Iowa City during an epidemic in 1945(5). This virus possessed little or no virulence for mice, produced no initial pulmonary consolidation, and did not maintain itself on serial passage in these animals. Cultivation by inoculation into the amniotic and chorioallantoic sacs of embryonated eggs was possible, although titers were low and irregular, rarely reaching an agglutination titer of 1-1600 against 0.25% chicken red blood cells. Virus from the second egg passage was available as seed. A third egg passage was carried out with a 1-3 dilution of the above virus using 11-day embryonated eggs inoculated into both amniotic and chorioallantoic sacs. A stock supply of this third passage virus, undiluted or mixed with an equal volume of hormone broth, was stored on dry ice in rubber-stoppered glass vials. It was not believed that adaptation to the egg had progressed very far, and the virus had never been in mice. This virus was diluted 1-3 or 1-5 and inoculated into eggs at the start of all tests to obtain sufficient virus for mouse inoculation. This undiluted fourth passage virus in chorioallantoic fluid was mixed with the adjuvant in the proportion of one part adjuvant to 2 parts virus for inoculation.

Bacteria used as adjuvants included *Hemophilus influenzae* type b, freshly isolated at the beginning of the experiment, a Group A beta hemolytic streptococcus, a type III *Diplococcus pneumoniae*, and *Vibrio comma*. Organism suspensions were prepared in concentrations comparable to a No. 2 barium sulfate standard. In addition various non-bacterial preparations of particulate nature

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1. Briody, B. A., *Bact. Rev.*, 1950, v14, 65.
2. Hirst, G. K., *J. Exp. Med.*, 1947, v86, 357.
3. Hirst, G. K., *J. Exp. Med.*, 1947, v86, 367.
4. Sugg, J. Y., *J. Bact.*, 1949, v57, 399.
5. McKee, Albert P., and Hale, Wm. M., *Science*, 1947, v105, 41.

were used, including fresh and heated chicken red blood cells, starch, kaolin, and powdered glass. Several preparations of soluble material were also used, including glycine extracts of bacterial cells prepared by the method of Cowles(6) and Maculla and Cowles(7), filtrates of frozen and thawed cells, purified polysaccharide of group† III pneumococcus, laked chicken red blood cells, and gum arabic.

Four-week-old Swiss mice were used as test animals; 4-6 animals were used for each series. Inoculation was made by the intranasal route, under ether anesthesia, using 0.1 ml inocula. With each test, half of the mice were observed for signs of infection over a period of 10 days, and half were used for passage on the third or fourth day, at which time pulmonary lesions were noted. Ten per cent lung suspension (2 parts) plus adjuvant (1 part) were used for passage in mice. Tests for the presence of virus in the mice were made by inoculation of 10% mouse lung, with added penicillin and streptomycin (1), into the amniotic and chorioallantoic sacs of 11-12-day embryonated eggs. Two passages were made at 35°C. Virus was considered present when chicken red blood cells were agglutinated by the allantoic fluid of the test eggs. Periodic neutralization tests in mice and hemagglutination inhibition tests with chicken red blood cells checked the identity of the virus.

Results. The unadapted virus never maintained itself alone in mouse lung, and was always absent after the third passage in mice. On the other hand, the IB45 virus was able to propagate in mouse lung when certain bacterial suspensions were added to the inocula at each mouse passage. For example, when *Hemophilus influenzae,* type b, cells were added either living, heat-killed (57°C for 60 minutes), or streptomycin-inactivated, virus was present for 4 passages and indefinitely thereafter. After 8-10 passages in mice, pulmonary lesions were more extensive, and deaths occurred in shorter times than before, providing evidence that the virus steadily increased in infectivity as it became adapted to the mouse. Once the virus became established in the mouse, the added factor could be omitted without losing the virus.

Group A beta hemolytic streptococci, type III *Diplococcus pneumoniae,* and *Vibrio comma* organisms could replace *Hemophilus influenzae* as factors stimulating virus propagation in mice. In all instances, once the virus maintained itself for 3 or 4 passages, it remained present.

Three per cent chicken red blood cells, or red cells heated at 60°C for 30 minutes also aided propagation of the virus in mouse lung. Laked fowl cells had no effect. A glycine extract of *Hemophilus influenzae,* type b, cells aided the growth of influenza B virus in mouse lung. Dialysis did not remove the active factor from the glycine extract, and when glycine was added alone to the virus in the amount used for cell extractions, it was ineffective. A filtrate of a 10-day broth culture of *Vibrio comma* could replace cells or the glycine extract. Another soluble preparation with which virus propagated was a purified Group III polysaccharide of *Diplococcus pneumoniae* used in 1% solution. The results of these experiments are summarized in Table I.

Summary and conclusions. The addition of a variety of bacterial cell suspensions, cell extracts, or non-bacterial substances to influenza virus, strain IB45, preparations at each passage in mice apparently aided the initially non-virulent virus in such a manner that it could establish itself in the mouse lung and propagate through repeated mouse passage. This addition of adjuvant had to be repeated at each passage for 4 to 8 passages, after which extensive lesions and regular deaths occurred in mice. At this time the adjuvant could be omitted and the virus would continue to propagate. Single addition of adjuvant at the first passage was not sufficient for the virus to establish itself in the mouse, since the virus disappeared after 3 passages. Virus alone was never present after the third passage in mice. There

6. Cowles, P. B., *Yale J. Biol. Med.,* 1947, v19, 835.

7. Maculla, Esther S., and Cowles, P. B., *Science,* 1948, v107, 376.

† This was a mixture of several types of polysaccharides, labeled Group III.

TABLE I. Effect of Adjuvants on the Adaptation of IB45 Virus to Mice.

Adjuvant	No. of trials	No. of passages	Virus
None	5	3,3,4,4,6	Ab
H. influenza, living cells, A*	2	3,27	P
" heat-killed cells, A	5	6,7,8,8,10	P
" streptomycin treated, A	1	8	P
" glycine extract, A	3	6,10,15	P
" glycine extract dialyzed, A	2	8,23	P
" living cells added once	1	3	Ab
" filtrate of cells frozen and thawed 20 times, A	1	8	Ab
D. pneumoniae, type III, heat-killed cells, A	1	7	P
Group A beta hemolytic streptococci, heat-killed, A	1	7	P
V. comma, boiled 1 hr, A	1	4	P
Pneumococcus Group III polysaccharide, A	1	6	P
V. comma, filtrate of 10-day broth culture, A	1	6	P
3% chicken red blood cells, A	2	4,8	P
" " " " " heated, A	2	4,8	P
Laked 3% chicken red blood cells, A	1	9	Ab
Powdered pyrex glass, A	1	8	P
Soluble starch, A	1	8	P
Kaolin, A	1	8	P
Glycine, A	1	4	Ab
1% gum arabic, A	1	8	Ab

A* = Added each passage. Ab = Absent. P = Present.

In the series in which living cells of *H. influenzae* were added, and 27 passages in mice made, the adjuvant was omitted after 4, 8, 12 and 20 passages, and the virus remained present from 5 to 15 passages thereafter; further passages were not made. Occasional titrations in mice showed infectivity titers of at least 10^{-4} to 10^{-5}.

In the series in which a dialyzed glycine extract was the adjuvant, it was discontinued after the 20th passage, and the virus continued to propagate; the animals died and showed lesions to the same extent as before.

is no evidence that any one of the preparations possessed greater action than the others, although the addition of killed bacterial cells seemed to give somewhat more rapid adaptation of the virus in the mouse than cell extracts or non-cellular substances. An influenza B virus that was initially non-infective for mice became infective when certain adjuvants of bacterial or non-bacterial origin were added to each passage of virus. A procedure such as this may be useful in the isolation and adaptation of influenza viruses in mice.

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Protective Effect of Salt on Reduction of Viscosity of Sodium Thymonucleate Solutions by Heat. (18182)

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The high viscosity of aqueous solutions of sodium thymonucleate, which is characteristic of the polymerized, asymmetric molecular state of this material, is readily reduced by a variety of agents. Among these are added salts(1,2), heat(1), changes in pH(3), and ultraviolet(4) and X-irradiation(5-8). The

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1. Hammarsten, E., *Biochem. Z.*, 1924, v144, 383.

2. Greenstein, J. P., *J. Nat. Cancer Inst.*, 1940, v1, 77.